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For: NOVEL METALLOPROTEASE HAVING AGGRECANASE ACTIVITY

STATEMENT

Assistant Commissioner for Patents
Alexandria, VA 22313-1450

Sir/Madam:

I, Katsunobu Ihara, residing at 306, 37-10, Shimizu 1-chome, Suginami-ku, Tokyo, Japan hereby state that:

I well understand the Japanese and English languages and attached is an accurate English translation made by me of Japanese Patent Application No. 2000-144020, filed May 16, 2000.

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[Title of the Invention] Novel Aggrecanase

[Claim]

[Claim 1] A metalloprotease which comprises an amino acid sequence of from the 213th position to the 583rd position of an amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

[Claim 2] A metalloprotease which comprises an amino acid sequence of from the 1st position to the 583rd position of an amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

[Claim 3] A metalloprotease having an aggrecanase activity, which consists of an amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

[Claim 4] A metalloprotease as described in any one of claims 1 to 3, which has an aggrecanase activity.

[Claim 5] A method for screening a substance capable of modifying the aggrecanase activity of a metalloprotease, which comprises allowing the metalloprotease as described in claim 4 to contact with a compound to be tested.

[Claim 6] A gene which encodes an amino acid sequence of the metalloprotease as described in any one of claims 1 to 3.

[Claim 7] A vector which comprises the gene described in claim 6.

[Claim 8] A host cell which comprises the vector described in claim 7.

[Claim 9] A method for producing the metalloprotease described in any one of claims 1 to 3, which comprises using the host cell described in claim 8.

[Claim 10] An antibody against the metalloprotease described in any one of claims 1 to 3,

[Claim 11] A method for screening a substance capable of modifying the aggrecanase activity of a metalloprotease, which comprises allowing the metalloprotease as described in any one of claims 1 to 3 to contact with a compound to be tested.

[Claim 12] An agent for inhibiting proteoglycan degradation, which comprises as an active ingredient a substance which inhibits metalloprotease described in claim 4.

[Claim 13] A gene represented by SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31, or a gene which is an equivalent of said gene.

[Detailed Description of the Invention]

[0001]

[Technical Field to which the Invention Belongs]

This invention relates to a novel metalloprotease, a gene coding for the metalloprotease, a method for producing the metalloprotease, a method for screening a substance capable of modifying the aggrecanase activity using the metalloprotease, an agent for inhibiting proteoglycan degradation, which comprises as an active ingredient a substance which inhibits a metalloprotease having an aggrecanase activity, and a promoter gene of the metalloprotease.

[0002]

[Prior Art]

Joint diseases are diseases which show damage and degeneration of joint cartilage as the main morbid states. Though a disease having the most frequent number of patients among joint diseases is osteoarthritis (OA), analgesic anti-inflammatory drugs and hyaluronic acid preparations are used in the current therapeutic method merely as a symptomatic therapy for the purpose of alleviating pains accompanied by the degeneration of cartilage and the destruction of bone under cartilage, so

that it cannot be said that they are exerting sufficient therapeutic effects.

Joint cartilage is a tissue mainly composed of type II collagen and aggrecan which is a cartilage-specific proteoglycan, and degradation and degeneration of both of them are observed in the joint diseases. Because of this, it has been considered for a long time that control of the degradation and degeneration of these extracellular matrix components would lead to the treatment of joint diseases, so that attempts have been positively made to identify degradation-concerned proteases (collagenase and aggrecanase) and to screen their inhibitors and develop them as medicaments.

As proteases having collagenase activities, matrix metalloproteases (MMP1, MMP8, MMP13, MMP14 and the like) have been identified, and their selective inhibitors have been discovered. However, in spite of the attempts to develop a large number of MMP inhibitors having collagenase inhibition activities as therapeutic drugs for joint diseases including OA and rheumatic arthritis (RA), MMP inhibitors to be used in these diseases as the indication have not been put on the market. Under such circumstances, attention has been directed toward aggrecanase which selectively degrades aggrecan which is another main constituting component of joint cartilage.

[0003]

A joint disease-related role of an enzyme aggrecanase which cleaves aggrecan at the site between Glu373-Ala374 has been revealed by the reports of Sandy *et al.* and Lohmander *et al.* stating that all of the main digested aggrecan fragments found in the synovial fluid of human arthritis patients were generated by cleaving at the aggrecanase digestion site (Sandy J.D. *et al.*, *J. Clin. Invest.*, 89, 1512 - 1516, 1992; Lohmander L.S. *et al.*, *Arthritis Rheum.*, 36, 1214 - 1222, 1993). On the other hand, it has been known that, in an *in vitro* explant culture system of joint cartilage, degradation of aggrecan firstly occurs by IL-1 induction and then degradation of type II collagen is accelerated (Dingle L.T. *et al.*, *Ann. Rheum. Dis.*, 34, 303 - 311, 1975; Cawston T.E. *et al.*, *Biochem. Biophys. Res. Comm.*, 215, 377 - 385, 1995; Kozaci L.D. *et al.*, *Arthritis Rheum.*, 40, 164 - 174, 1997). It has been reported that the aggrecan degradation takes the precedence of the type II collagen degradation in a mouse arthritis model too (van Meurs J.B. *et al.*, *Arthritis Rheum.*, 42, 1128 - 1139, 1999). These reports suggest a possibility that the type II collagen degradation can be controlled by inhibiting the preceding aggrecan degradation.

[0004]

However, the entity of the aggrecanase has been unclear for long time, though its biochemical properties

had been elucidated, namely it is a metalloprotease, it exists in outside of cells, a glycosaminoglycan side chain is concerned in its substrate recognition, its activity is induced by IL-1, TNF and retinoic acid, and the like. Recently, ADAMTS4 (aggrecanase-1: Tortorella M.D. *et al.*, *Science*, 284, 1664 - 1666, 1999) and ADAMTS11 (aggrecanase-2: Abbaszade I. *et al.*, *J. Biol. Chem.*, 274, 23443 - 23450, 1999) have been reported as proteases having an aggrecanase activity. However, because their gene expression in human OA cartilage is not increased, and their gene expression in an *in vitro* explant culture system of human knee joint cartilage is not induced by IL-1, TNF and retinoic acid which induce the aggrecanase activity that causes joint diseases, existence of another protease relating to joint deseases was suggested. (Flannery C.R. *et al.*, *Biochem. Biophys. Res. Commun.*, 260, 318 - 322, 1999).

[0005]

[Problems that the Invention is to Solve]

The objects of the invention are to provide recombinant proteins by isolating and identifying genes coding for aggrecanases which are concerned in the onset and progress of joint diseases, particularly osteoarthritis as a disease having the most frequent number of patients and thereby constructing their expression production systems, to enable screening of a substance capable of modifying the aggrecanase activity by constructing a

screening system of the substance, and to show proteoglycan degradation inhibition action of the substance.

[0006]

[Means for Solving the Problems]

Under such circumstances, the present inventors have conducted intensive studies and succeeded as a result in isolating a gene coding for a novel protein classified into the ADAMTS family, determining its complete ORF sequence and thereby enabling production of a recombinant protein.

Also, a vector containing this gene, a host cell containing this vector and a method for producing the novel protein using this host cell were established. Based on these, screening of compounds, peptides and antibodies capable of modifying protease activity of the protein was enabled and the invention was accomplished.

In addition, it was found that this protein has the activity to cut the extracellular substrate aggrecan selectively between Glu373-Ala374, namely an aggrecanase activity as one of the protease activities, which suggested a possibility of the use of a compound capable of significantly modifying this protein and activity of the protein as a medicament.

Thereafter, the invention has been accomplished by finding a compound which significantly inhibits aggrecanase activity of this protein and showing that this compound has

an action to inhibit degradation of proteoglycans in a system which uses joint cartilage primary culture cells.

[0007]

Accordingly, the invention relates to

(1) a metalloprotease which comprises an amino acid sequence of from the 213th position to the 583rd position of an amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

(2) a metalloprotease which comprises an amino acid sequence of from the 1st position to the 583rd position of an amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

(3) a metalloprotease having an aggrecanase activity, which consists of an amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or a metalloprotease which is an equivalent of said metalloprotease.

(4) a metalloprotease as described in any one of (1) to (3), which has an aggrecanase activity.

(5) a method for screening a substance capable of modifying the aggrecanase activity of a metalloprotease, which comprises allowing the metalloprotease as described in (4) to contact with a compound to be tested.

(6) a gene which encodes an amino acid sequence of the metalloprotease as described in any one of (1) to (3).

(7) a vector which comprises the gene described in (6).

(8) a host cell which comprises the vector described in (7).

(9) a method for producing the metalloprotease described in any one of (1) to (3), which comprises using the host cell described in (8).

(10) an antibody against the metalloprotease described in any one of (1) to (3),

(11) a method for screening a substance capable of modifying the aggrecanase activity of a metalloprotease, which comprises allowing the metalloprotease as described in any one of (1) to (3) to contact with a compound to be tested.

(12) an agent for inhibiting proteoglycan degradation, which comprises as an active ingredient a substance which inhibits metalloprotease described (4).

(13) a gene represented by SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31, or a gene which is an equivalent of said gene.

[0008]

[Mode for Carrying Out the Invention]

The following describes the terms used in the invention.

The "metalloprotease" as used herein means a "metalloprotease" which has a zinc coordination consensus sequence (HExxH) and has a protease activity. Also, unless otherwise noted, the "protease" is referred to as "protein".

The novel metalloprotease of the invention is any of a metalloprotease which contains an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and has a protein degradation ability, or a metalloprotease which is an equivalent of the metalloprotease.

Also, the novel metalloprotease of the invention any of a metalloprotease which contains an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and has a protein degradation ability, or a metalloprotease which is an equivalent of the metalloprotease.

In addition, it is any of a metalloprotease which has the amino acid sequence represented by SEQ ID NO:1, an

amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and has a protein degradation ability, or a metalloprotease which is an equivalent of this metalloprotease.

Regarding the "equivalent of the metalloprotease", (1) in the case of an equivalent of the metalloprotease containing an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 213th position to the 583rd position, and which has the protein degradation ability, (2) in the case of an equivalent of the metalloprotease containing an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the

1st position to the 583rd position, and which has the protein degradation ability, or (3) in the case of an equivalent of the metalloprotease containing the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in respective sequences, and which has the protein degradation ability. Preferred as the "equivalent of the metalloprotease" is the metalloprotease formed by amino acid substitution such as SNP.

Preferred as the novel metalloprotease of the invention are a polypeptide having the amino acid sequence represented by SEQ ID NO:1, a polypeptide having an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, a polypeptide having an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 and a polypeptide having an amino acid sequence of from the 213th position to

the 583rd position of the amino acid sequence represented by SEQ ID NO:1, particularly a polypeptide having an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 and a polypeptide having an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1.

[0009]

The gene coding for the novel metalloprotease of the invention is any gene which contains a nucleotide sequence coding for the metalloprotease, namely a metalloprotease gene which contains a nucleotide sequence coding for an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 or an equivalent of the metalloprotease and which has a protein degradation ability.

Also, the gene coding for the novel metalloprotein of the invention may be any metalloprotease gene which contains a nucleotide sequence coding for a metalloprotease represented by an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1 or for an equivalent of the metalloprotease and which has a protein degradation ability.

In addition, it may be any metalloprotease gene which has a nucleotide sequence coding for a metalloprotease

represented by the amino acid sequence shown in SEQ ID NO:1, or by an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence shown in SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1, or for an equivalent of the metalloprotease, and which has a protein degradation ability.

Regarding the "nucleotide sequence coding for an equivalent of the metalloprotease", (1) in the case of a nucleotide sequence coding for an equivalent of the metalloprotease containing an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 213th position to the 583rd position, and which has the protein degradation ability, (2) in the case of a nucleotide sequence coding for an equivalent of the metalloprotease containing an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein

degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 1st position to the 583rd position, and which has the protein degradation ability, or (3) in the case of a nucleotide sequence coding for an equivalent of the metalloprotease containing the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in respective sequences, and which has the protein degradation ability. Preferred as the "nucleotide sequence coding for an equivalent of the metalloprotease" is a nucleotide sequence coding for the metalloprotease formed by amino acid substitution such as SNP.

Regarding the gene coding for the novel metalloprotease of the invention, it is preferably a gene

having from the 1st position to the 1749th position, from the 1st position to the 2850th position, from the 637th position to the 1749th position or from the 637th position to the 2850th position, of the nucleotide sequence described in SEQ ID NO:2, more preferably a gene having from the 637th position to the 1749th position or from the 637th position to the 2850th position, of the nucleotide sequence described in SEQ ID NO:2.

A promoter gene is included in the invention, and the promoter gene of the invention is preferably a gene having a nucleotide sequence described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31. In the "gene as an equivalent of the gene described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31", one to several bases may be substituted, deleted and/or inserted at one to several positions in the nucleotide sequence described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31.

The novel metalloprotease of the invention has various protease activities, and it is a particularly noteworthy point that it has an aggrecanase activity as one of these activities.

Also, the metalloprotease of the invention having the aggrecanase activity can be used in the screening of substances capable of modifying the aggrecanase activity. Among substances capable of modifying the metalloprotease having aggrecanase activity, a substance which inhibits the

aggrecanase activity is useful as a proteoglycan degradation inhibitor. In addition, the promoter gene of the metalloprotease of the invention exists in two or more variant forms. Accordingly, it is a noteworthy point that a substance capable of modifying the promoter activity can be screened using a desired variant of the promoter gene in response to each purpose.

[0010]

Now, a gene coding for the novel protein of the invention, a vector of the invention, a host cell of the invention, a method for producing the novel protein of the invention, a method for detecting activity of the novel protein of the invention, a method for producing an antibody which reacts with the novel protein of the invention, a method for screening a substance capable of modifying activity of the novel protein of the invention, a method for detecting promoter activity and a method for screening a substance capable of modifying the promoter activity are described in the following 1) to 9). All of the items described in 1) to 9) are included in the invention.

[0011]

<u>1) Production method of protein gene</u>

a) First production method - a method which uses PCR

A mRNA sample is extracted from a human cell or tissue having the ability to produce the novel protein of

the invention. Next, using this mRNA as the template, two primers interposing the mRNA or a part of mRNA of the novel protein are prepared. Full-length cDNA or a part thereof corresponding to the novel protein can be obtained by modifying denature temperature, denaturing agent adding condition and the like and carrying out a reverse transcriptase-polymerase chain reaction (to be referred to as RT-PCR hereinafter) suited for a respective protein comprising a part of the amino acid sequence represented by SEQ ID NO:1 of the invention. Alternatively, full-length cDNA or a part thereof corresponding to the novel protein can be obtained by carrying out a polymerase chain reaction (to be referred to as RT-PCR hereinafter), by using cDNA prepared using reverse transcriptase from mRNA which is extracted from a human cell or tissue having the ability to produce the novel protein of the invention, or a commercially available cDNA preparation derived from a human cell or tissue, as the template. Thereafter, the novel protein can be produced by integrating the thus obtained full-length cDNA or a part thereof corresponding to the novel protein into an appropriate expression vector and expressing it in a host cell.

Firstly, mRNA comprising a sequence coding for the protease is extracted from a human cell or tissue having the ability to produce the novel protein of the invention by a known method. As the extraction method, a guanidine

thiocyanate hot phenol method, a guanidine thiocyanate-guanidine hydrochloride method and the like can be exemplified, but a guanidine thiocyanate cesium chloride method can be preferably cited. The cell or tissue having the ability to produce this protease can be specified by, e.g., northern blot technique using a gene or a part thereof having a nucleotide sequence coding for the protease or western blot technique using an antibody specific for the protease.

Purification of mRNA can be carried out in accordance with a usual method, for example, it can be purified by binding it to an oligo(dT) cellulose column and then eluting it. Alternatively, a commercially available extracted and purified mRNA may be used without extracting the mRNA.

Subsequently, single-strand cDNA is synthesized by carrying out reverse transcriptase reaction of the purified mRNA in the presence of random primers, oligo(dT) primers or custom-synthesized primers. Using two primers interposing a part of the gene of interest, the thus obtained single-strand cDNA is subjected to PCR to amplify the novel protein DNA of interest. Alternatively, a commercially available cDNA preparation may be used without synthesizing the cDNA. The thus obtained DNA is fractionated by a means such as agarose gel electrophoresis or the like. If desired, a DNA fragment of interest can be

obtained by digesting this DNA with restriction enzymes and the like and then ligating the digested fragments.

[0012]

b) Second production method

In addition to the above production method, the gene of the invention can be produced using conventional genetic engineering techniques. Firstly, single-strand cDNA is synthesized using reverse transcriptase and using the mRNA obtained by the above method as the template and then double-strand cDNA is synthesized from this single-strand cDNA. As the method, the S1 nuclease method (Efstratiadis, A. *et al.*, *Cell*, 7, 279 - 288, 1976), Land method (Land, H. *et al.*, *Nucleic Acids Res.*, 9, 2251 - 2266, 1981), O. Joon Yoo method (Yoo, O.J. *et al.*, *Proc. Natl. Acad. Sci. USA*, 79, 1049 - 1053, 1983), the Okayama-Berg method (Okayama, H. and Berg, P., *Mol. Cell. Biol.*, 2, 161 - 170, 1982) and the like can be exemplified.

Next, an *Escherichia coli* strain such as DH5 α strain, HB101 strain, JM109 strain or the like is transformed by introducing a recombinant plasmid obtained by the aforementioned method, and a resulting recombinant can be selected using the resistance for a drug such as tetracycline, ampicillin, kanamycin or the like as a marker. Transformation of a host cell, for example, when the host cell is *E. coli*, can be carried out by Hanahan's method (Hanahan, D.J., *Mol. Biol.*, 166, 557 - 580, 1983),

namely, by adding the recombinant DNA to competent cells prepared in the coexistence of $CaCl_2$ and $MgCl_2$ or RbCl. As a matter of course, commercially available competent cells can also be used. In this connection, in addition to a plasmid, a phage vector such as a lambda system can also be used as a vector.

Regarding the method for selecting DNA of the novel protein of interest from the thus obtained transformants, various methods shown below can for example be employed.

[0013]

(i) A screening method which uses a synthetic oligonucleotide probe

An oligonucleotide corresponding to whole or a part of the novel protein of the invention is synthesized (in this case, it may be either a nucleotide sequence derived by using the codon usage or a combination of two or more possible nucleotide sequences, and in the latter case, the number of their kinds can be reduced by including inosine), this is hybridized as a probe (after labeling with ^{32}P or ^{33}P) with a nitrocellulose filter or nylon filter on which DNA samples of the transformants are denatured and immobilized, and then the thus obtained positive strains are screened and selected.

(ii) A screening method which uses a probe prepared by polymerase chain reaction

Oligonucleotides of a sense primer and an antisense primer corresponding to a part of the novel protein of the invention are synthesized and polymerase chain reaction (Saiki, R.K. *et al.*, *Science*, 239, 487 - 491, 1988) is carried out using these primers, thereby effecting amplification of a DNA fragment coding for whole or a part of the novel protein of interest. As the template DNA to be used, cDNA synthesized by reverse transcription reaction from mRNA of cells producing the novel protein or genomic DNA can be used. The thus prepared DNA fragment is labeled with ^{32}P or ^{33}P and used as the probe to carry out colony hybridization or plaque hybridization to select the clone of interest.

(iii) A screening method in which the novel protein is produced by other animal cells

A transformant is cultured to amplify a gene, an animal cell is transfected with the gene (in this case, the vector may be either an autonomously replicating plasmid comprising a transcription promoter region or a plasmid which can be integrated into chromosome of the animal cell), and the protein encoded by the gene is produced in the extracellular moiety. By detecting the novel protein using an antibody specific for the novel protein of the invention, a strain comprising cDNA which encodes the novel

protein of interest is selected from the original transformants.

(iv) A selection method which uses an antibody specific for the novel protein of the invention

By integrating cDNA into an expression vector in advance, proteins are produced in culture supernatants, inside the cells or on the surface of cells of transformants, and the strain of interest is selected by detecting the novel protein producing strain of interest using an antibody specific for the novel protein of the invention and a secondary antibody against this antibody.

(v) A method which uses a selective hybridization-translation system

Samples of cDNA obtained from transformants are blotted on a nitrocellulose filter or the like, mRNA prepared from the novel protein producing cells of the invention is hybridized therewith, and then the mRNA hybridized to the cDNA is dissociated and recovered. The thus recovered mRNA samples are translated into proteins in a protein translation system, e.g., a system in which they are injected into oocyte of Xenopus or a cell free system such as rabbit reticulocyte lysate, wheat germ or the like. The strain of interest is selected by detecting it using an antibody against the novel protein of the invention.

The method for collecting DNA coding for the novel protein of the invention from the thus obtained

transformant of interest can be carried out in accordance with gene manipulation experiment manuals such as of a known method (Sambrook, J. *et al.*, "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989) and the like. For example, it can be achieved by separating a fraction corresponding to plasmid DNA from cells and then cutting out the cDNA region from the plasmid DNA.

[0014]

c) Third production method

The novel protein gene of the invention can also be produced by connecting DNA fragments produced by a chemical synthesis method. Each DNA can be synthesized using a DNA synthesizing machine [e.g., Oligo 1000M DNA Synthesizer (mfd. by Beckman), 394 DNA/RNA Synthesizer (mfd. by Applied Biosystems) or the like].

[0015]

d) Fourth production method

The novel protein gene of the invention can also be produced based on the information on the novel protein, for example, by chemical synthesis of nucleic acids in accordance with a conventional method such as phosphite triester method (Hunkapiller, M. *et al.*, *Nature*, 10, 105 - 111, 1984) or the like. In this connection, codons for desired amino acids are well known, can be selected optionally and can be determined in accordance with a

conventional method (Crantham, R. *et al.*, *Nucleic Acids Res.*, 9, r43 - r74, 1981), taking codon usage of the host to be used into consideration. In addition, partial modification of codons of these nucleotide sequences can be carried out in the usual way in accordance with the site specific mutagenesis (Mark, D.F. et al., Proc. Natl. Acad. Sci. USA, 81, 5662 - 5666, 1984) or the like which uses primers comprised of synthetic oligonucleotides which encode the desired modification.

[0016]

Determination of sequences of DNA obtained by the above methods a) to d) can be carried out for example by Maxam-Gilbert chemical modification method (Maxam, A.M. and Gilbert, W., "Methods in Enzymology", 65, 499 - 559, 1980), dideoxy nucleotide chain termination method (Messing, J. and Vieira, J., *Gene*, 19, 269 - 276, 1982) and the like.

The vector of the present invention, the hose cell of the present invention, and the novel protein of the present invention are obtainable by the following methods.

[0017]

2) Methods for the production of the vector of the invention, the host cell of the invention and the recombinant protein of the invention

The thus isolated fragment containing the gene coding for the novel protein of the invention can be transformed into eucaryotic or procaryotic host cells by again

integrating it into an appropriate vector DNA. In addition, it is possible to express the gene in respective host cells by introducing an appropriate promoter and a sequence concerned in the gene expression into these vectors.

For example, the eucaryotic host cells include cells of a vertebrate, an insect, yeast and the like, and COS cell as a monkey cell (Gluzman, Y., *Cell*, 23, 175 - 182, 1081), a dihydrofolate reductase deficient strain of Chinese hamster ovary cell (CHO) (Urlaub, G. and Chasin, L.A., *Proc. Natl. Acad. Sci. USA*, 77, 4216 - 4220, 1980), human fetal kidney-derived HEK293 cell, 293-EBNA cell in which Epstein-Barr virus EBNA-1 gene is introduced into the same cell (mfd. by Invitrogen) and the like are frequently used as the vertebrate cells, though limited thereto.

As the expression vector for vertebrate cells, a vector having a promoter, a RNA splicing site, a polyadenylylation site, a transcription termination sequence and the like generally positioned upstream of the gene to be expressed can be used, and it may further have a replication origin as occasion demands. Examples of the expression vector include pSV2dhfr having SV40 early promoter (Subramani, S. *et al.*, *Mol. Cell. Biol.*, 1, 854 - 864, 1981), pEF-BOS having human elongation factor promoter (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18,

5322, 1990), pCEP4 having cytomegalovirus promoter (mfd. by Invitrogen) and the like, though not limited thereto.

In the case of the use of COS cell as the host cell, an expression vector which has SV40 replication origin, can perform autonomous replication in COS cell and has a transcription promoter, a transcription termination signal and an RNA splicing site can be used, and its examples include pME18S (Maruyams, K. and Takebe, Y., *Med. Immunol.*, 20, 27 - 32, 1990), pEF-BOS (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18, 5322, 1990), pCDM8 (Seed, B., *Nature*, 329, 840 - 842, 1987) and the like. The expression vector can be incorporated into COS cell by a DEAE-dextran method (Luthman, H. and Magnusson, G., *Nucleic Acids Res.*, 11, 1295 - 1308, 1983), a calcium phosphate-DNA co-precipitation method (Graham, F.L. and van der Ed, A.J., *Virology*, 52, 456 - 457, 1973), a method which uses FuGENE™6 Transfection Reagent (mfd. by Boehringer Mannheim), an electroporation method (Neumann, E. *et al.*, *EMBO J.*, 1, 841 - 845, 1982) and the like, thus enabling to obtain a desired transformant cell.

[0018]

Also, when CHO cell is used as the host cell, a transformant cell which can stably produce the novel protein can be obtained by co-transfecting a vector capable of expressing neo gene which functions as a G418 resistance marker, such as pRSVneo (Sambrook, J. *et al.*, "Molecular

Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989), pSV2-neo (Southern, P.J. and Berg, P., *J. Mol. Appl. Genet.*, 1, 327 - 341, 1982) or the like, together with an expression vector and then selecting a G418-resistant colony. Also, when 293-EBNA cell is used as the host cell, a desired transformant cell can be obtained using an expression vector which has Epstein-Barr virus replication origin and can perform autonomous replication in the 293-EBNA cell, such as pCEP4 (mfd. by Invitrogen) or the like.

The thus obtained transformant cell of interest can be cultured in accordance with a conventional method, and the novel protein of the invention is produced in extracellular moiety by this culturing. As the medium to be used in the culturing, various conventionally used media can be optionally selected depending on the host cell employed. In the case of, for example, the COS cell, a medium such as a RPMI-1640 medium, Dulbecco's modified Eagle's minimum essential medium (DMEM) or the like which may be supplemented, as occasion demands, with a serum component such as fetal bovine serum (FBS) or the like may be used. Also, in the case of the 293-EBNA cell, a medium such as Dulbecco's modified Eagle's minimum essential medium (DMEM) or the like supplemented with a serum component such as fetal bovine serum (FBS) or the like and further supplemented with G418 may be used.

The novel protein of the invention thus produced in the extracellular moiety of the transformant cell can be separated and purified by various known separation techniques making use of physical characteristics, biochemical characteristics and the like of the novel protein. Illustrative examples of such techniques include treatment of a culture broth containing the novel protein with a usual protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, high performance liquid chromatography (HPLC) and the like, dialysis and combinations thereof.

When the novel protein of the invention is expressed after its in frame fusion with a marker sequence, expression verification, purification and the like of the novel protein become possible. Examples of the marker sequence include FLAG epitope, Hexa-Histidine tag, Hemagglutinin tag, myc epitope and the like. Also, when a specific amino acid sequence recognizable by proteases such as enterokinase, factor Xa, thrombin and the like is inserted between a marker sequence and the novel protein, the marker sequence moiety can be cut off and removed by these proteases.

[0019]

3) Method for detecting metalloprotease activity of the novel protein of the invention

Protease activity of the novel protein of the invention and a partial peptide thereof can be detected by mixing the novel protein of the invention or a partial peptide thereof with a substrate cited below in an appropriate buffer solution, allowing them to undergo the reaction and then detecting it by a method suited for respective substrate.

As the substrate, a fluorescence- or radiation-labeled substrate, a synthetic substrate having a fluorophore, a quenching group or a chromophore, an unlabeled substrate and the like can be exemplified. Examples of the fluorescence- or radiation-labeled substrate include fluorescence- or radiation-labeled gelatin, collagen, synthetic peptides and the like, the synthetic substrate having a fluorophore includes Glt-Ala-Ala-Phe-MCA, Lys-MCA, Pyr-Arg-Thr-Lys-Arg-MCA and the like (Peptide Research Institute), the synthetic substrate having a quenching group includes MOCAc-Arg-Pro-Lys-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met(Dnp)-NH_2, MOCAc-Tyr-Val-Ala-Asp-Ala-Pro-Lys(Dpn)-NH_2 and the like (Peptide Research Institute), the synthetic substrate having a chromophore includes Ala-pNA, Bz-Tyr-pNA, Pyr-Phe-Leu-pNa and the like (Peptide Research Institute) and the unlabeled substrate includes casein, collagen, fibronectin, aggrecan, gelatin

and the like proteins, insulin and the like physiologically active peptides, synthetic peptides and the like. The synthetic peptides include those which contain non-natural type amino acids too.

For example, when a radiation-labeled substrate or a substrate having a fluorophore, a quenching group or a chromophore is used, the protease activity can be detected using a liquid scintillation counter, a fluorescence detector, a spectrophotometer or the like appropriate detector. When an unlabeled substrate is used, the protease activity can be detected by analyzing its degradation product using SDS-PAGE, HPLC, Zymography or the like.

[0020]

4) Method for detecting aggrecanase activity

As the substrate for detecting aggrecanase activity, aggrecan purified from a cartilage or tissue of human or other animal, aggrecan obtained by genetic recombination, commercially available aggrecan (Seikagaku Kogyo) or a partial protein thereof can be used. The aggrecanase activity can be measured by allowing these substrates to react with a cell or tissue culture broth, cell or tissue extract or (partially) purified sample containing a protease to be tested, and then detecting a fragment cleaved off between Glu373-Ala374. For the detection of the fragment cleaved off between Glu373-Ala374, a method in which

an N-terminal sequence or a C-terminal sequence of the digested fragment is determined in accordance with a conventional method, or more conveniently, ELISA (enzyme-linked immunosorbent assay) which uses an anti-neoepitope antibody (Hughes C.E. *et al.*, *Biochem. J.*, 305, 799 - 804, 1995) capable of specifically recognizing C-terminal NITGE and N-terminal ARGSV formed by the cutting between Glu373-Ala374, a western blotting or the like immunological method can be used.

[0021]

5) Method for preparing antibody which reacts with the novel protein of the invention

The antibody which reacts with the novel protein of the invention, such as a polyclonal antibody or a monoclonal antibody, can be obtained by directly administering the novel protein or a fragment of the novel protein to various animals. It can also be obtained by a DNA vaccine method (Raz, E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 91, 9519 - 9523, 1994; Donnelly, J.J. *et al.*, *J. Infect. Dis.*, 173, 314 - 320, 1996) using a plasmid into which a gene coding for the novel protein of the invention is introduced.

A polyclonal antibody is produced from a serum or egg of an animal such as rabbit, rat, goat, domestic fowl or the like which is sensitized by immunizing the animal with the novel protein or a fragment thereof emulsified in an

appropriate adjuvant such as complete Freund's adjuvant by its peritoneal, subcutaneous, intravenous or the like injection. The thus produced polyclonal antibody can be separated and purified by usual protein isolation and purification techniques, and examples of the usual protein isolation and purification techniques include centrifugation, dialysis, salting out with ammonium sulfate and a chromatography using DEAE-cellulose, hydroxyapatite, protein A agarose or the like.

A monoclonal antibody can be produced easily by those skilled in the art by the cell fusion method of Kohler and Milstein (Kohler, G. and Milstein, C., *Nature*, 256, 495 - 497, 1975).

That is, mouse is immunized by emulsifying the novel protein of the invention or a fragment thereof in an appropriate adjuvant such as complete Freund's adjuvant and inoculating the emulsion several times at intervals of a few weeks by its peritoneal, subcutaneous, intravenous or the like injection. After the final immunization, spleen cells are taken out and fused with myeloma cells to prepare hybridomas.

As the myeloma cells for obtaining hybridomas, a myeloma cell having a marker (e.g., hypoxanthine-guanine phosphoribosyl transferase deletion, thymidine kinase deletion or the like), such as a mouse myeloma cell strain P3X63Ag8.U1, is used. Also, polyethylene glycol is used as

the fusing agent. As the medium for preparing hybridomas, Eagle's minimum essential medium, Dulbecco's minimum essential medium, RPMI1640 or the like usually used medium is used by optionally supplementing it with 10 to 30% fetal bovine serum. The fused strains are selected by the HAT selection method. Screening of hybridomas is carried out using culture supernatants by ELISA, immunological tissue staining or the like well known method or by the aforementioned screening method, and a clone of hybridoma which secretes the antibody of interest is selected. Also, monoclonal nature of the hybridoma is confirmed by repeating subcloning by limiting dilution. By culturing the thus obtained hybridoma in a medium for several days or in the abdominal cavity of a pristane-pretreated BALB/c mouse for 10 to 20 days, the antibody is produced in a purification-possible amount. The thus produced monoclonal antibody can be separated and purified from the culture supernatant or ascitic fluid by usual protein isolation purification techniques.

Active antibody fragments containing a part of the antibody, such as $F(ab')_2$, Fab, Fab' and Fv, can be obtained by digesting the thus separated and purified antibody with a proteolytic enzyme such as pepsin, papain or the like in the conventional way and then separating and purifying the fragments by usual protein isolation purification techniques.

Furthermore, it is possible to obtain the antibody which reacts with the novel protein of the invention as single chain Fv or Fab by the methods of Clackson *et al.* and Zebedee *et al.* (Clackson, T. *et al.*, *Nature*, 352, 624 - 628, 1991; Zebedee, S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 3175 - 3179, 1992). In addition, it is possible to obtain a human antibody by immunizing a transgenic mouse in which a mouse antibody gene is replaced by a human antibody gene (Lonberg, N. et al., *Nature*, 368, 856 - 859, 1994).

[0022]

6) Method for screening a substance capable of modifying metalloprotease activity of the novel protein of the invention

The screening method of the invention contains a means in which, using the novel protein prepared at least by the production methods shown by the aforementioned 1) and 2), a substance to be tested is added to a system for measuring an index of the modification of the metalloprotease of the novel protein in response to a biochemical characteristic of the novel protein, and the index is measured. In this case, examples of the measuring system include various well-known protease measuring systems (Biochemical Experimentation Methods 30 "Proteolytic Enzymes I" edited by D. Tsuru and M. Funatsu published by Gakkai Shuppan Center, 1993, ibid. 31, "Proteolytic Enzymes I" Gakkai Shuppan Center, 1993), and

screening of the substance to be tested can be carried out by, in accordance with or applying the treating methods described in the documents.

Regarding the substance to be tested, compounds or peptides which are generally known to have metalloprotease inhibition activity but their activities to modify metalloprotease activity of the novel protein are unclear, or various known compounds and peptides, compounds synthesized using combinatorial chemistry techniques (Terrett, N.K. *et al.*, *Tetrahedron*, 51, 8135 - 8137, 1995) or general synthesis techniques and random peptides prepared by applying a phage display method (Felici, F. *et al.*, *J. Mol. Biol.*, 222, 301 - 310, 1991) can be used. In addition, extracts and culture supernatants of microorganisms, natural components derived from plants and marine organisms, animal tissue extracts and the like are also become objects of the screening. Alternatively, compounds or peptides prepared by chemically or biologically modifying the compounds or peptides selected by the screening method of the invention can also be used.

For the screening of substances (compounds, peptides, antibodies and antibody fragments) capable of modifying protease activity of the novel protein of the invention , any substance which becomes the substrate of the novel protein of the invention or of a partial peptide thereof

can be used, and the substrates described in the aforementioned 3) are desirable.

[0023]

7) Method for screening a substance capable of modifying aggrecanase activity of the novel protein of the invention

This can be screened by a similar method of the aggrecanase activity detection method described in 4). Also, the ELISA or the like method exemplified in (Example 10-2) can be used, in which added aggrecan, recombinant aggrecan, commercially available aggrecan or a partial protein thereof which disappears or decreases by its degradation when allowed to react with the novel protein of the invention is measured using an antibody which specifically recognizes polypeptides of the N-terminal side and C-terminal side moieties of the region cut off with aggrecanase. Also useful is a method in which the novel protein of the invention is allowed to react with a recombinant aggrecan in which FLAG tag is added to the N-terminal, and His tag to the C-terminal, as exemplified in (Example 7-1), and the added recombinant aggrecan disappeared or decreased by its degradation is measured by ELISA or the like method using an anti-FLAG tag and anti-His tag antibodies. The tags in this case are not limited to FLAG tag and His tag, and the recombinant aggrecan is not limited to (Example 7-1) and may be a partial protein or modified protein of aggrecan which is cut off at the

aggrecanase cutting site by this protein. As the test substances to be used for the aggrecanase activity, substances similar to the test substances to be used for the metalloprotease activity of 6) are used.

[0024]

8) Method for detecting promoter activity

As the method for detecting the promoter activity possessed by the sequences and partial sequences thereof shown in Examples, a method in which a reporter gene plasmid is used is convenient. The reporter gene means a gene coding for a protein which can be determined by usual means (e.g., measurement of enzyme activities and the like determination methods well known to those skilled in the art), and chloramphenicol acetyltransferase, luciferase, β-galactosidase and alkaline phosphatase genes are frequently used, though not limited thereto. Regarding a vector as the base for constructing a reporter gene plasmid, there is no limitation and commercially available plasmid vectors such as pGV-B2 (mfd. by Toyo Ink), pSEAP2-Basic (mfd. by Clontech) and the like can be used. By constructing a reporter gene plasmid in which the sequence is inserted in the forward direction into upstream of the reporter gene of these vectors and measuring amount of the reporter protein expressed in cells transformed with this plasmid, by a method suited for respective case, the presence and strength of the promoter activity of the sequence can be

known, and action of a substance to be tested upon this promoter activity can be detected by adding the substance to be tested to a culture broth of the transformed cells.

[0025]

9) Method for screening a substance capable of modifying the promoter activity of the invention

For the screening of substances (compounds, peptides, antibodies and antibody fragments) which inhibit the promoter activity possessed by the sequence of the Sequence ID No. of the invention and a partial sequence thereof, a method similar to the promoter activity detection method shown in 8) can be used. Regarding the substance to be tested, compounds or peptides which are generally known to modify promoter activity but their activities to modify the promoter activity possessed by the sequences of SEQ ID NOs;24 to 31 and partial sequences thereof are unclear, or various known compounds and peptides, compounds synthesized using combinatorial chemistry techniques (Terrett, N.K. *et al.*, *Tetrahedron*, 51, 8135 - 8137, 1995) or general synthesis techniques and random peptides, antibodies and antibody fragments prepared by applying a phage display method (Felici, F. *et al.*, *J. Mol. Biol.*, 222, 301 - 310, 1991) can be used. In addition, extracts and culture supernatants of microorganisms, natural components derived from plants and marine organisms, animal tissue extracts and the like are also become the object of the screening.

Alternatively, compounds or peptides prepared by chemically or biologically modifying the compounds or peptides selected by the screening method of the invention can also be used.

[0026]

A medicament which comprises as the active ingredient the novel protein or a substance, among the substances (compounds, peptides, antibodies or antibody fragments) capable of significantly modifying activities of the novel protein selected by the aforementioned screening method, capable of significantly inhibiting the activity of a metalloprotease having aggrecanase activity is included in the invention, and a proteoglycan degradation inhibitor is particularly desirable as the medicament. Examples of the substance which significantly inhibits activity of the novel protein include known compounds selected by the screening system shown in (Example 10-2) and disclosed in WO 90/05719, but not only medicaments containing these known compounds as the active ingredient but also all medicaments which contain substances capable of significantly inhibiting activity of the novel protein are included in the invention. In this connection, regarding the production method of the known compounds disclosed in WO 90/05719, they can be synthesized in accordance with the production method disclosed in this International Publication.

The pharmaceutical preparations which comprise as the active ingredient the novel protein of the invention or a substance (a compound, peptide, antibody or antibody fragment) which significantly inhibits activity of the novel protein can be prepared using carriers, fillers and other additives usually used for their preparation, in response to each type of the active ingredient.

Examples of its administration include oral administration using tablets, pills, capsules, granules, fine subtilaes, powders, oral solutions and the like and parenteral administration using intravenous, intramuscular, intraarticular and the like injections, suppositories, percutaneous preparations, transmucosal preparations and the like. Particularly in the case of peptides which are digested in the stomach, parenteral administration such as intravenous injection or the like is desirable.

In the solid composition for use in the oral administration according to the present invention, one or more active substances are mixed with at least one inert diluent such as lactose, mannitol, glucose, microcrystalline cellulose, hydroxypropylcellulose, starch, polyvinyl pyrrolidone, aluminum magnesium silicate or the like. In the usual way, the composition may contain other additives than the inert diluent, such as a lubricant, a disintegrating agent, a stabilizing agent, a solubilizing or solubilization assisting agent or the like. If

necessary, tablets or pills may be coated with a sugar or a film of a gastric or enteric substance.

[0027]

The liquid composition for oral administration includes emulsions, solutions, suspensions, syrups and elixirs and contains a generally used inert diluent such as purified water or ethanol. In addition to the inert diluent, this composition may contain auxiliary agents such as a moistening agent, a suspending agent, a sweetener, an aromatic agent and an antiseptic agent.

The injections for parenteral administration includes aseptic aqueous or non-aqueous solutions, suspensions and emulsions. Examples of the diluent for use in the aqueous solutions and suspensions include distilled water for injection, physiological saline and the like. Examples of the diluent for use in the non-aqueous solutions and suspensions include propylene glycol, polyethylene glycol, plant oil (e.g., olive oil or the like), alcohol (e.g., ethanol), Polysorbate 80 and the like. Such a composition may further contain a moistening agent, an emulsifying agent, a dispersing agent, a stabilizing agent, a solubilizing or solubilization assisting agent, an antiseptic and the like. These compositions are sterilized by filtration through a bacteria retaining filter, blending of a germicide or irradiation. Alternatively, they may be used by firstly making into sterile solid compositions and

dissolving them in sterile water or a sterile solvent for injection use prior to their use.

The clinical dose is optionally decided by taking into consideration strength of activity of the active ingredient selected by the aforementioned screening method, symptoms, age, sex and the like of each patient to be treated.

[0028]

[Examples]

The following describes the invention more illustratively.

Unless otherwise noted, experiments were carried out in accordance with gene manipulation experiment manuals such as of a known method (Sambrook, J. *et al.*, "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989) and the like, but the invention is not limited to the Examples.

[0029]

(Example 1) Discovery of partial sequence of a novel ADAMTS gene MDTS6

A human brain cDNA library strictly fractionated by the size of insertion sequences was constructed as shown in a reference (Ohara O. *et al.*, *DNA Res.*, 4, 53 - 59, 1997). Size distribution of cDNA fragments in these sub-libraries was from 3 kbp to 8 kbp. By deciphering 5′- and 3′-end sequences of clones constituting this library, an in-house

EST data bank was constructed. A partial sequence of MDTS6 was obtained from this.

[0030]

(Example 2) Determination of full-length ORF sequence of MDTS6

By determining sequences of MDTS6 cDNA clones, a sequence of from the 832nd position to the 2853rd position of SEQ ID NO:2 was obtained. The sequence of from the 1st position to the 831st position of SEQ ID NO:2 was obtained by repeating RACE (Rapid Amplification of cDNA Ends) using human brain and human placenta Marathon-Ready™ cDNA manufactured by Clontech as the template and LA-Taq™ (mfd. by Takara Shuzo) as the DNA polymerase. As a result, it was revealed that the full-length MDTS6 was a novel protein composed of 950 amino acids as shown in SEQ ID NO:1. Its domain structure was composed of a secretion signal sequence, a pro region, a furin protease recognition sequence, a metalloprotease domain, a disintegrin domain, a thrombospondin type I repeat sequence (to be referred to as TSP-1 repeat sequence hereinafter), a domain rich in Cys residue, an intermediate region and two TSP-1 repeat sequences, in order from the N-terminus, and it was a molecule belonging to the ADAMTS family (Kuno, K. *et al.*, *J. Biol. Chem.*, 272, 556 - 562, 1997; Tang, B.L. *et al.*, *FEBS Lett.*, 445, 223 - 225, 1999).

[0031]

(Example 3) Preparation of C-terminal FLAG addition type expression vector

An EBNA1 expression unit-removed expression vector pCEP4d was constructed by digesting pCEP4 (mfd. by Invitrogen) with restriction enzymes *Cla*I and *Nsi*I, blunt-ending the resulting fragments and then carrying out their autonomous ligation. This vector was digested with restriction enzymes *Nhe*I and *Bam*HI and extracted from an agarose gel to obtain a fragment of about 7.7 kbp, and a double strand of oligonucleotide obtained by annealing a nucleic acid shown by SEQ ID NO:3 and a nucleic acid shown by SEQ ID NO:4 was inserted into the fragment to select a clone having the planned sequence which was named pCEP4d-FLAG. Using this vector as the template and oligoDNA shown by SEQ ID NO:5 and oligoDNA shown by SEQ ID NO:6 as primers, PCR was carried out using PyroBest™ DNA polymerase. The thus generated DNA fragment of about 0.4 kbp was digested with a restriction enzyme *Spe*I and inserted into pCEP4d-FLAG (about 7.7 kbp) which had been digested with *Xba*I, and a clone in which *Xba*I, *Nhe*I, *Not*I and *Bam*HI recognition sequence cloning sites and FLAG tag were arranged in that order from the promoter as intended was selected, thereby completing pCEP4dE2-FLAG.

[0032]

(Example 4) Construction of MDTS6 truncated protein (MDTS6TSP1) expression plasmid

A plasmid was constructed in the following manner for use in expressing a sequence of from the 1st position to the 583rd position of SEQ ID NO:1 (a moiety corresponding to a region containing the TSP1 repeat sequence from the N-terminus of MDTS6 (to be referred to as MDTS6TSP1 hereinafter)) as a protein in which FLAG was added to the C-terminus.

Firstly, a gene of from the 1st position to the 1749th position of SEQ ID NO:2 was obtained by PCR. Using oligoDNA sequences represented by SEQ ID NO:7 and SEQ ID NO:8 as primers, human placenta Marathon-Ready™ cDNA (mfd. by Clontech) as the template and LA-Taq™ (mfd. by Takara Shuzo) as DNA polymerase, a cycle of 98°C for 10 seconds and 68°C for 2 minutes was repeated 10 times after heating at 94°C for 1 minutes. Using a DNA solution prepared by 50 times-diluting this reaction solution as the template and using PyroBest™ DNA polymerase, PCR was carried out under a condition of 94°C for 2 minutes, 40 repetitions of a cycle of 98°C for 10 seconds, 66°C for 30 seconds and 74°C for 4 minutes and subsequent 72°C for 10 minutes. The thus generated fragment of interest in which *Xba*I recognition sequence and Kozak sequence were added to the 5′ side, and *Not*I recognition sequence to the 3′ side, was subcloned into PCR-Blunt to confirm the sequence and then digested

with restriction enzymes *Xba*I and *Not*I and inserted into the *Xba*I-*Not*I site of pCEP4dE2-FLAG to complete pCEP-MDTS6TSP1-FLAG.

[0033]

(Example 5) Construction of MDTS6 full-length protein expression plasmid

A plasmid was constructed in the following manner for use in expressing a sequence of from the 1st position to the 950th position of SEQ ID NO:1 as a protein in which FLAG was added to the C-terminus.

Firstly, a gene of from the 1534th position to the 2850th position of SEQ ID NO:2 was obtained by PCR. Illustratively, using oligoDNA sequences represented by SEQ ID NO:9 and SEQ ID NO:10 as primers, the plasmid DNA of EST clone as the template and PyroBest™ DNA polymerase as DNA polymerase, a cycle of 98°C for 10 seconds, 50°C for 15 seconds and 72°C for 2 minutes was repeated 20 times after heating at 94°C for 1 minutes, followed by 7 minutes of reaction at 72°C. The thus generated fragment of interest in which *Not*I recognition sequence was added to the 3′ side was subcloned into PCR-Blunt to confirm the sequence and used as pCRB-MDTS6-3H.

Making use of the presence of a *Bam*HI recognition sequence in a sequence of from the 1566th position to the 1571st position of SEQ ID NO:2, pCEP-MDTS6TSP1-FLAG was digested with restriction enzymes *Xba*I and *Bam*HI, and the

thus generated DNA fragment of about 1.6 kbp was connected to a DNA fragment of about 1.3 kbp generated by digesting pCRB-MDTS6-3H with *Bam*HI and *Not*I and inserted into the *Xba*I-*Not*I site of pCEP4dE2-FLAG to complete pCEP-MDTS6F-FLAG.

[0034]

(Example 6) Expression of MDTS6TSP1 by animal cell strain

The expression plasmid prepared in Example 4 using pCEP4dE2-FLAG as the backbone was introduced into HEK293-EBNA cell (mfd. by Invitrogen) using FuGENE™6 Transfection Reagent (mfd. by Boehringer Mannheim) in accordance with the attached instructions. After introduction of the plasmid, the presence of the protein of interest in a culture supernatant obtained by 1 to 2 days of culturing was confirmed by western blotting using an antibody against FLAG tag added to the C-terminus (a mouse anti-FLAG monoclonal antibody (M2; mfd. by Sigma)). That is, the culture supernatant was subjected to electrophoresis using SDS/10% to 20% acrylamide gel (mfd. by Daiichi Pure Chemicals) and then transferred on a PVDF membrane using a blotting apparatus. The PVDF membrane after the transfer was subjected to blocking by adding Block Ace (mfd. by Dainippon Pharmaceutical) and then allowed to react with the mouse anti-FLAG monoclonal antibody (M2; mfd. by Sigma) and a horseradish peroxidase-labeled rabbit anti-mouse IgG

polyclonal antibody (mfd. by Zymed or TAGO) in that order. Alternatively, after the blocking, it was allowed to react with biotinylated M2 antibody (mfd. by Sigma) and a streptoavidine-horseradish peroxidase conjugate (mfd. by Amersham) in that order. After the reaction, expression of the protein was confirmed using an ECL western blotting detection system (mfd. by Amersham Pharmacia) (Fig. 1). Molecular weight of the expressed MDTS6TSP1 protein was smaller than the value calculated from the amino acid sequence by a factor of about 23 K. Making use of the fact that FLAG tag is added to the C-terminus of MDTS6TSP1 protein expressed by the HEK293-EBNA cell as described in the foregoing, MDTS6TSP1 protein was affinity-purified by the method of Example 7-1 and then transferred on a PVDF membrane, and the N-terminal sequence of MDTS6TSP1 protein stained with Ponceau S was determined by analyzing with Type 494 Peptide Sequencer manufactured by ABI. As a result, it was shown that it starts from the 213th position Phe of SEQ ID NO:1 and, similar to the case of other ADAMTS molecules, becomes mature protein (from 213th position to 583rd position of SEQ ID NO:1) by being cleaved at the furin protease recognition sequence existing between the pro region and metalloprotease domain. Also, the MDTS6 full-length protein was obtained in the same manner as the case of the above MDTS6TSP1 protein expression using the expression plasmid obtained in Example 5, and similar to

the case of MDTS6TSP1, it was shown that it becomes mature protein (from 213th position to 950th position of SEQ ID NO:1) by being cleaved at the furin protease recognition sequence existing between the pro region and metalloprotease domain.

[0035]

(Example 7) Detection of enzyme activity of MDTS6TSP1 protein expressed in animal cell host

(Example 7-1) Preparation of recombinant aggrecan G1G2

Using oligoDNA sequences represented by SEQ ID NO:11 and SEQ ID NO:12 synthesized based on the reported gene sequence of human aggrecan (Doege K. *et al.*, *Biochem. Soc. Trans.*, 18, 200 - 202, 1990) as primers, human placenta Marathon-Ready™ cDNA as the template and PyroBest™ DNA polymerase as DNA polymerase, the reaction of 94°C for 1 minute, 40 repetitions of a cycle of 98°C for 10 seconds and 68°C for 2 minutes, and subsequent 68°C for 7 minutes was carried out. The thus generated DNA fragment was digested with a restriction enzyme *Bam*HI and inserted into the *Bam*HI site of pCEP-SigFla, thereby completing an expression plasmid pCEP-rAgg for use in the expression of a protein in which FLAG tag is added to the N-terminus, and His tag to the C-terminus, of the globular domain 1 (G1)-globular domain 2 (G2) of human aggrecan. The pCEP-SigFla is an expression vector which is prepared by introducing

double strand of the oligoDNA sequences represented by SEQ ID NO:13 and SEQ ID NO:14 into the *Hin*dIII-*Xho*I site of pCEP4d and has the influenza virus hemagglutinin secretion signal sequence described in the report (Guan X-M. *et al.*, *J. Biol. Chem.*, 267, 21995 - 21998, 1992), FLAG tag and *Bam*HI recognition sequence in that order downstream of the promoter.

The plasmid pCEP-rAgg was introduced into HEK293-EBNA cell which was subsequently cultured for 3 to 7 days, thereby effecting expression and production of the protein of interest. Purification of the protein of interest from the culture supernatant was carried out by an affinity purification making use of the addition of FLAG tag to the N-terminus. That is, the culture supernatant was applied to M2-agarose (mfd. by Sigma) packed in a column, washed with 20 mM Tris-HCl (pH 7.4)/150 mM NaCl (to be referred to as TBS hereinafter), eluted and fractionated with 0.1 M Gly-HCl (pH 3.0) and immediately neutralized with 1 M Tris-HCl (pH 8.0).

[0036]

(Example 7-2) Detection of recombinant aggrecan G1G2 degrading activity of MDTS6 truncated protein

In Example 6, the medium 12 to 16 hours after introduction of the expression plasmid was replaced by a serum-free medium, and the culturing was continued for 32 to 36 hours to recover the culture supernatant. This

culture supernatant was mixed with the recombinant aggrecan prepared in the foregoing, and the mixture was allowed to undergo the reaction at 37°C overnight, subjected to SDS-PAGE, transferred on a PVDF membrane and blocked by the method described in Example 6 and then allowed to react with an anti-Hisx6 polyclonal antibody (sc-803; mfd. by Santa Cruz Biotechnology) and a horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (mfd. by MBL) in that order. After the reaction, the recombinant aggrecan was detected using an ECL western blotting system (mfd. by Amersham Pharmacia). As a result, degraded fragment of the recombinant aggrecan, which was not found in the control in which only the expression plasmid was introduced, was detected (Fig. 2).

[0037]

(Example 7-3) Analysis by anti-aggrecanase neoepitope antibody

Aggrecanase is a metalloprotease which selectively cleaves aggrecan at the site between Glu373-Ala374. An antibody capable of recognizing a C-side neoepitope generated by this cleavage was prepared in accordance with a usual method by repeating immunization of mouse with a conjugate of the synthetic peptide having Ala-Arg-Gly-Ser-Val-Val-Leu-Thr-Ala-Lys-Cys and KLH, 5 times. A PVDF membrane after transfer and blocking carried out in the same manner as in Example 7-2 was allowed to react with

this antibody, allowed to react with a peroxidase-labeled goat anti-mouse IgG polyclonal antibody (mfd. by Tago) and then detected using an ECL western blotting detection system (mfd. by Amersham Pharmacia). As a result, the degraded product of recombinant aggrecan generated by MDTS6 reacted with the anti-aggrecanase neoepitope antibody, and molecular weight of the detected band is consistent with the molecular weight of the degraded product detected in Example 7-2 (Fig. 3). The same result was obtained by the BC-3 antibody which recognizes aggrecanase neoepitope (Hughes C.E. et al., Biochemical J., 305, 799 - 804, 1995).

[0038]

(Example 8) Expression induction of MDTS6 mRNA by IL-1

It is known that a mouse cell strain ATDC5 is differentiated into a chondrocyte-like cell by insulin treatment (Atsumi T. et al., Cell Differ. Dev., 30, 109 - 116, 1990). The ATDC5 cells were inoculated in 4 x 10^5/well portions into an I type collagen-coated 6 well plate (mfd. by Asahi Technoglass) and cultured for 2 days using DMEM/HamF12 (1:1)/5% FCS medium, the medium was changed to DMEM/HamF12 (1:1)/5% FCS medium containing insulin (final concentration 30 ng/ml) and 50 μg/ml of L-ascorbic acid and the culturing was continued for 5 days, and then the resulting cells were treated for 0, 1, 2, 4 or 8 hours by adding IL-1β (final concentration 5 ng/ml).

Total RNA was prepared from each of the treated groups using ISOGEN (mfd. by Nippon Gene), and RT-PCR was carried out using 1 μg portion thereof as the template and using BcaBEST™ RNA PCR Kit (mfd. by Takara Shuzo). The reverse transcription reaction was carried out using Oligo dT-Adaptor Primer as the primer in accordance with the attached instructions, and PCR was carried out using oligoDNA sequences represented by SEQ ID NO:15 and SEQ ID NO:16 as primers, which had been synthesized based on the 3′ non-translation region of MDTS6, by the reaction of 94°C for 2 minutes, 40 repetitions of a cycle of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and subsequent 72°C for 7 minutes. The reaction solution was subjected to electrophoresis with 1% agarose, and densities of the thus generated bands of about 0.3 kbp were compared. As a result, it was found that expression of the MDTS6 mRNA is transiently induced by IL-1 (Fig. 4).

[0039]

(Example 9) Degradation of natural type aggrecan by MDTS6

(Example 9-1) Expression of various full-length MDTS6 proteins and their recombinant aggrecan G1G2 degrading activity

The expression plasmid constructed using pCEP4dE2-FLAG as the backbone was introduced into HEK293-EBNA cell (mfd. by Invitrogen) using FuGENE™6 Transfection Reagent

(mfd. by Boehringer Mannheim) in accordance with the attached instructions. After introduction of the plasmid, the resulting cells were cultured overnight and washed with PBS buffer, and then the medium was changed to a serum-free medium and the culturing was continued for 2 to 3 days. The resulting culture broth was centrifuged at 9,000 rpm for 10 minutes, and the supernatant was used as the enzyme source of MDTS6. In this case, in addition to the expression plasmids described in Example 4 and Example 5, expression plasmids for three proteins, namely a protein in which the polypeptide of Gly-Ser-Ala-Ala-Ala-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys was added to the C-terminus of the amino acids of from the 1st position to the 447th position of SEQ ID NO:1, of the amino acids of from the 1st position to the 518th position of SEQ ID NO:1, of the amino acids of from the 1st position to the 685th position of SEQ ID NO:1, of the amino acids of from the 1st position to the 841st position of SEQ ID NO:1, or of the amino acids of from the 1st position to the 896th position of SEQ ID NO:1, were designed as expression plasmids. For example, the expression plasmid for a protein in which the polypeptide of Gly-Ser-Ala-Ala-Ala-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys was added to the C-terminus of the amino acids of from the 1st position to the 685th position of SEQ ID NO:1 (hereinafter, referred to as MDTS6Cys), which was used in the following Examples, was constructed by amplifying a gene by PCR using

the full-length protein expression plasmid constructed in Example 5 as the template and the oligoDNA sequences represented by SEQ ID NO:7 and SEQ ID NO:17 as primers and using PyroBest DNA polymerase, digesting the gene with restriction enzymes *Xba*I and *Not*I, and then inserting the resulting fragment into the *Xba*I-*Not*I site of pCEP4dE2-FLAG.

The aggrecanase activity of the various MDTS6 proteins above were evaluated by the method of Example 7-3. As a result, no aggrecanase activity was found in the protein having amino acids of from the 1st position to the 447st position of SEQ ID NO:1 and in the protein having amino acids of from the 1st position to the 518st position of SEQ ID NO:1. That is, it was revealed that the first TSP-1 repeat sequence counting from the N-terminus is essential for exerting the aggrecanase activity of MDTS6. Further, it was revealed that the ratio of the protein secreted to the supernatant of the culture was low in the case of the latter two proteins and the full-length protein.

[0040]

(Example 9-2) Degradation of natural type aggrecan

A 90 μl portion of the MDTS6 enzyme solution prepared in Example 9-1 was mixed with a solution of 10 μg natural type aggrecan (mfd. by Seikagaku Kogyo)/10 μl TBS in a test tube and allowed to undergo the reaction at 37°C overnight.

This reaction product was dried up using SpeedVac and then dissolved in 100 μl of 10 mM Tris-acetate buffer (pH 7.6) containing 0.06 unit of Chondroitinase ABC (mfd. by Seikagaku Kogyo), 0.024 unit of keratanase I (mfd. by Seikagaku Kogyo), 0.0004 unit of keratanase II (mfd. by Seikagaku Kogyo), 5 μM of PMSF and 10 mM of EDTA, and the solution was allowed to undergo the reaction at 37°C overnight. A portion of this reaction solution was subjected to SDS-PAGE and then the product was detected using the mouse anti-aggrecanase neoepitope antibody as shown in Example 7-3. In this case, the peroxidase-labeled goat anti-mouse IgG polyclonal antibody used was a preparation manufactured by Biosource. The same result was obtained by the BC-3 antibody which recognizes aggrecanase neoepitope (Hughes C.E. et al., Biochemical J., 305, 799 - 804, 1995).

As a result, a band of 80 to 90 KDa was detected in the case of MDTS6Cys in addition to a band of about 150 KDa. This degradation pattern is consistent with the pattern of main molecules (all generated by aggrecanase degradation) found in the joint synovial fluids of patients of joint diseases including OA and RA (Sandy J.D. *et al.*, *J. Clin. Invest.*, 89, 1512 - 1516, 1992; Lohmander L.S. *et al.*, *Arthritis Rheum.*, 36, 1214 - 1222, 1993) and also is consistent with the pattern of main molecules having aggrecanase neoepitope which are generated after 12 to 24

hours of treatment with IL-1 and retinoic acid in an explant culture system of human knee joint cartilage (Little C.B. *et al.*, *Biochemical J.*, 344, 61 - 68, 1999) (Fig. 5).

[0041]

(Example 10) Screening system of substances capable of modifying the aggrecanase activity

(Example 10-1) Preparation of MDTS6Cys and substrate

It was confirmed using the western blotting method shown in Example 9-2 that the recombinant aggrecan G1G2 and the natural type aggrecan are cleaved off at the aggrecanase site by MDTS6Cys without purification but as the culture supernatant prepared by the method of Example 9-1. Also, the cleavage at the "aggrecanase site" was observed when the culturing in Example 9-1 was continued with the 10% FBS-containing medium without changing to the serum-free medium. Accordingly, the recombinant aggrecan G1G2 prepared in Example 7-1 was used as the substrate.

[0042]

(Example 10-2) Screening system

Though the screening can be carried out by the western blotting-aided method shown in Example 7-2 using the rAgg-G1-G2 or natural type aggrecan as the substrate, the following ELISA system was constructed for screening more larger number of compounds to be tested.

An MDTS6Cys culture supernatant, the recombinant aggrecan G1G2 and a compound to be tested were mixed and allowed to undergo the reaction at 37°C for several hours, the resulting product was adhered to a 96 well plate (Nunc-Immuno™ Plate MaxiSorp™ Surface # 439454; mfd. by Nunc), blocked with 1% BSA/TBS solution and then allowed to react with a mouse anti-aggrecanase neoepitope antibody and an HRP-anti-mouse IgG antibody conjugate (mfd. by Biosource) in that order, and then the detection was carried out using TMB Peroxidase EIA Substrate Kit (mfd. by Bio-Rad) to calculate the aggrecanase activity inhibiting strength of the compound to be tested using the coloring inhibition as a marker. Also, as a modified method thereof, rAggG1-G2 was adhered to the 96 well plate (the same as above) and blocked with 1% BSA/TBS solution in advance and then an MDTS6Cys culture supernatant and a compound to be tested were added thereto and allowed to undergo the reaction at 37°C for several hours, the resulting product was allowed to react with a mouse anti-aggrecanase neoepitope antibody and an HRP-anti-mouse IgG antibody conjugate (mfd. by Biosource) in that order, and then the detection was carried out using TMB Peroxidase EIA Substrate Kit (mfd. by Bio-Rad) to calculate the aggrecanase activity inhibiting strength of the compound to be tested using the coloring inhibition as a marker. By using this screening system, it was able to select N-α-[3-(N-hydroxycarbamoyl)-2-isobutyl-

3-mercaptopropionyl]-N-o-dimethyltyrosineamide (Compound A) and N-α-[3-(N-hydroxycarbamoyl)-2-isobutyl-4-mercaptopropionyl]-N-methylphenylalanineamide (Compound B).

[0043]

(Example 11)

(Example 11-1) Preparation of rabbit knee joint cartilage primary culture cells

After killing a rabbit (Japanese white species, male, 1.0 to 1.5 kg) under excess anesthesia, a knee joint was excised and the cartilage layer on the joint surface was removed and finely cut using a surgical knife. The cut pieces were treated with trypsin-EDTA (0.25%-1 mM; mfd. by GIBCO-BRL) at 37°C for 1 hour and then centrifuged at 1,500 rpm for 5 minutes, and the resulting precipitate was washed with DMEM. This was treated with collagenase A (0.15%; Boehringer-Mannheim)/DMEM at 37°C for 3 to 4 hours, and then a nylon mesh filter (100 μm, mfd. by Falcon)-passed fraction was centrifuged at 1,500 rpm for 5 minutes to effect precipitation of cartilage cells. The cells were thoroughly washed with DMEM/10% FBS medium, suspended in DMEM/10% FBS medium to a density of 2×10^5 cells/ml and then inoculated in 200 μl/well portions into an I type collagen-coated 96 well plate (mfd. by Asahi Technoglass). Three days thereafter, the medium was changed to 200 μl of DMEM/10% FBS medium containing 50 μg/ml of ascorbic acid (ascorbic acid medium hereinafter), and the culturing was

continued for 3 days. When an I type collagen-coated 6 well plate (Asahi Technoglass) was used, the cell suspension was inoculated in 6 ml/well portions and cultured by carrying out the same medium exchange. These cells were used in the following test.

[0044]

(Example 11-2) Proteoglycan degradation of rabbit knee joint cartilage primary culture cells

The rabbit knee joint cartilage primary culture cells of 96 well plate described in Example 11-1 were cultured for 2 days using 200 μl of the ascorbic acid medium supplemented with 10 μCi/ml in final concentration of $Na_2{}^{35}SO_4$ and labeled therewith, washed 3 times with 200 μl of the ascorbic acid medium and then cultured for 1 day using 200 μl of the ascorbic acid medium. After stimulation with IL-1β or all-trans retinoic acid and subsequent 0, 24, and 48 hours of culturing, the culture supernatants were recovered in 20 μl portions, and the radioactivity was measured using Top Count (mfd. by Packard). As a result, increase in the radioactivity, namely release of proteoglycan, was observed by 0.01 to 10 ng/ml of IL-1β stimulation, and increase in the concentration-dependent and strong radioactivity, namely release of proteoglycan, was observed by 0.1 to 10 μM of all-trans retinoic acid stimulation (Fig. 6).

[0045]

(Example 11-3) Induction of MDTS6 mRNA expression

After 3 days of culturing of the rabbit knee joint cartilage primary culture cells of 6 well plate described in Example 11-1 by changing the medium to ascorbic acid medium, 10 ng/ml of IL-1β or 10 μM of all-trans retinoic acid was added thereto, and total RNA samples 2 and 6 hours thereafter were prepared using ISOGEN (mfd. by Nippon Gene) in accordance with the attached instructions. Each of the samples was treated with DNase I (mfd. by Nippon Gene), subjected to phenol/chloroform treatment and then recovered by ethanol precipitation, and the thus purified total RNA was dissolved in DEPC-treated sterile water. Using random hexamers as primers, 1 μg of this total RNA was subjected to reverse transcription reaction and RNase H treatment using Thermoscript™ RT-PCR System (mfd. by GIBCO-BRL, catalog number 11146-016) in accordance with the attached instructions, and the product was diluted 10 times with sterile water and used as a cDNA sample. Using 5 μl of each of the thus obtained cDNA samples as the template and the oligoDNA sequences represented by SEQ ID NO:18 and SEQ ID NO:19 as primers, PCR was carried out under a condition of 94°C for 2 minutes, 45 repetitions of a cycle of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds, and subsequent 72°C for 10 minutes. The reaction products were subjected to 2% agarose electrophoresis, and

densities of the generated DNA fragments were compared. As a result, expression of the MDTS6 mRNA was induced by IL-1β and all-trans retinoic acid, and the expression strength correlated with the degree of proteoglycan degradation described in Example 11-2 (Fig. 7).

[0046]

(Example 12) Inhibition of proteoglycan degradation in rabbit knee joint cartilage primary culture cells by substances which inhibit the aggrecanase activity

Each of the compounds A and B selected by the screening system of Example 10-2 was added to the proteoglycan degradation system of rabbit knee joint cartilage primary culture cells just before the stimulation with 10 μM of all-trans retinoic acid, and their proteoglycan degradation inhibitory activities were examined. As a result, the compounds A and B showed the inhibition of proteoglycan degradation in a concentration-dependent manner (Fig. 8). On the other hand, the proteoglycan degradation inhibition action was not observed by compounds which have the same hydroxamic acid backbone but show a weak aggrecanase activity inhibition, even at a concentration of 100 μM.

[0047]

(Example 13) Analysis of MDTS6 promoter region DNA sequence

A DNA fragment corresponding to the promoter region of MDTS6 was amplified using PCR from GenomeWalker DNA Sca I Libraries (Genome Walker™ Kits, CLONTECH catalog number K1803-1). OligoDNA sequences of the adapter primers AP-1 (SEQ ID NO:20) and AP-2 (SEQ ID NO:21) attached to the kit were used as forward primers, and the oligoDNA sequences of SEQ ID NO:22 and SEQ ID NO:23 as reverse primers. The illustrative method was as described in the instructions attached to the kit, but TAKARA LA Taq (TAKARA LA Taq™, catalog number RR002A) was used in the PCR. The first PCR was carried out using the oligoDNA sequences of SEQ ID NO:20 and SEQ ID NO:22 as primers under a condition of 7 repetitions of a cycle of 98°C for 5 seconds and 72°C for 3 minutes, 32 repetitions of a cycle of 98°C for 5 seconds and 67°C for 3 minutes, and 67°C for 4 minutes. The second PCR was carried out under the same conditions using 5 μl of a solution prepared by diluting the reaction solution of the first reaction 50 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) as the template, and the oligoDNA sequences of SEQ ID NO:21 and SEQ ID NO:23 as primers. When the thus amplified DNA fragment of about 3.7 kbp was directly subjected to sequence analysis by dideoxy terminator method using ABI377 DNA Sequencer (Applied Biosystems Inc.), DNA sequences of about 2.2 kbp, 0.36 kbp and 0.8 kbp divided by two un-decipherable gaps were found.

Next, in order to decipher sequences of these two gap moieties which were unable to decipher by the direct analysis of the PCR-amplified DNA fragment, this DNA fragment was subcloned and the DNA nucleotide sequence was determined. In this connection, pZErO™-2 vector (Zero Background/Kan Cloning Kit, mfd. by Invitrogen, catalog number K2600-01) was used as the cloning vector, and the subcloning was carried out in accordance with the attached instructions.

A plasmid prepared by inserting the above DNA fragment into the *Kpn*I-*Xho*I site of a reporter plasmid pGV-B2 (mfd. by Toyo Ink) was introduced into HEK293 cell using FuGene-6, and the luciferase activity after 28 or 48 hours of culturing under usual culturing conditions was measured using PicaGene coloring kit (mfd. by Toyo Ink, catalog number PGK-L100). In this case, the measured value was normalized by the activity value of β-gal expressed by a simultaneously introduced β-gal expression plasmid pCH110 (Amersham Pharmacia Biotech, catalog number 27-4508-01). The β-gal activity was measured using Galacto-Light Plus Kit (mfd. by TROPIX, catalog number BL300P). As a result, distinct increase in the luciferase activity which cannot be found in the original plasmid pGV-B2 was observed. This result indicates that the promoter activity is present in the above DNA fragment.

[0048]

[Effects of the Invention]

The novel metalloprotease obtained by the invention is characterized in that, since it has a protease activity, it can be used as a medicament and for the screening of a substance (a compound, a peptide, an antibody or an antibody fragment) to be used in a medicament which significantly modifies activity of the protease. In this case, regarding the medicinal use of the protease and a substance which significantly modifies activity of the protease, it is effective for diseases which are caused by acceleration, reduction, degeneration and the like abnormalities of the activity of the novel protease or in which the abnormalities are expressed to cause complications, such as cancer, arthritis, osteoarthritis and the like. Also, the metalloprotease of the invention is characterized in that, since it has aggrecanase activity, it can be used for the screening of the metalloprotease having aggrecanase activity and a substance (a compound, a peptide, an antibody or an antibody fragment) which significantly modifies the metalloprotease having aggrecanase activity. In this case, as the medical use of the metalloprotease having aggrecanase activity and a substance which significantly modifies the metalloprotease having aggrecanase activity, it is suggested that it is effective in preventing and treating diseases which are caused by acceleration, reduction,

degeneration and the like abnormalities of the activity of the novel protease or in which the abnormalities are expressed to cause complications, particularly joint diseases as diseases which show proteoglycan degradation, most particularly osteoarthritis.

[Brief Description of the Drawings]

[Fig. 1] Fig. 1 is a photograph showing a result of the expression of MDTS6TSP1 in an animal cell strain using an ECL western blotting detection system, obtained in Example 6.

[Fig. 2] Fig. 2 is a photograph showing a result of the detection of the activity of MDTS6TSP1 to degrade a recombinant aggrecan G1G2 using an ECL western blotting detection system, obtained in Example 7-2.

[Fig. 3] Fig. 3 is a photograph showing a result of the analysts of a recombinant aggrecan G1G2 degraded with MDTS6TSP1, by an anti-aggrecanase neoepitope antibody, using a western blotting detection system, obtained in Example 7-3.

[Fig. 4] Fig. 4 is an electrophoresis pattern photograph showing a result of the examination of MDTS6 mRNA expression induction by IL-1β, obtained in Example 8.

[Fig. 5] Fig. 5 is a photograph showing a result of the detection of degradation of natural type aggrecan by MDTS6 protein, by an anti-aggrecanase neoepitope antibody,

using a western blotting detection system, obtained in Example 9-2.

[Fig. 6] Fig. 6 is a graph showing a result of the detection of release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid and IL-1β, obtained in Example 11-2.

[Fig. 7] Fig. 7 is an electrophoresis pattern photograph showing a result of the analysis of changes in gene expression of MDTS6 by RT-PCR when rabbit knee joint primary culture cells are treated with all-trans retinoic acid and IL-1β, obtained in Example 11-3.

[Fig. 8] Fig. 8 is a graph showing that degradation and release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid are inhibited by the compound A and compound B, obtained in Example 12.

[Designation of Document] Abstract

[Abstract]

[Problem] To provide a recombinant protein which is necessary for isolating and identifying genes coding for novel ADAMTS proteins having a markedly high possibility as drug creation target molecules and for screening substances capable of modifying their activities.

[Means for Resolution] A gene coding for a novel protein classified into the ADAMTS family was isolated, its complete ORF sequence was determined and the protein was expressed. A vector containing this gene, a host cell containing this vector, a method for producing the protein using this host cell and a method for screening this protein and compounds, peptides and antibodies capable of modifying activity of the protein were established.

[Selected Drawing] None

[Sequence Listing]

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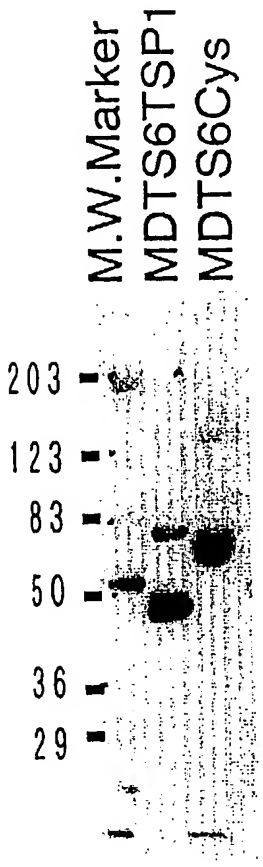
[Designation of Document] Drawings

[Fig. 1]



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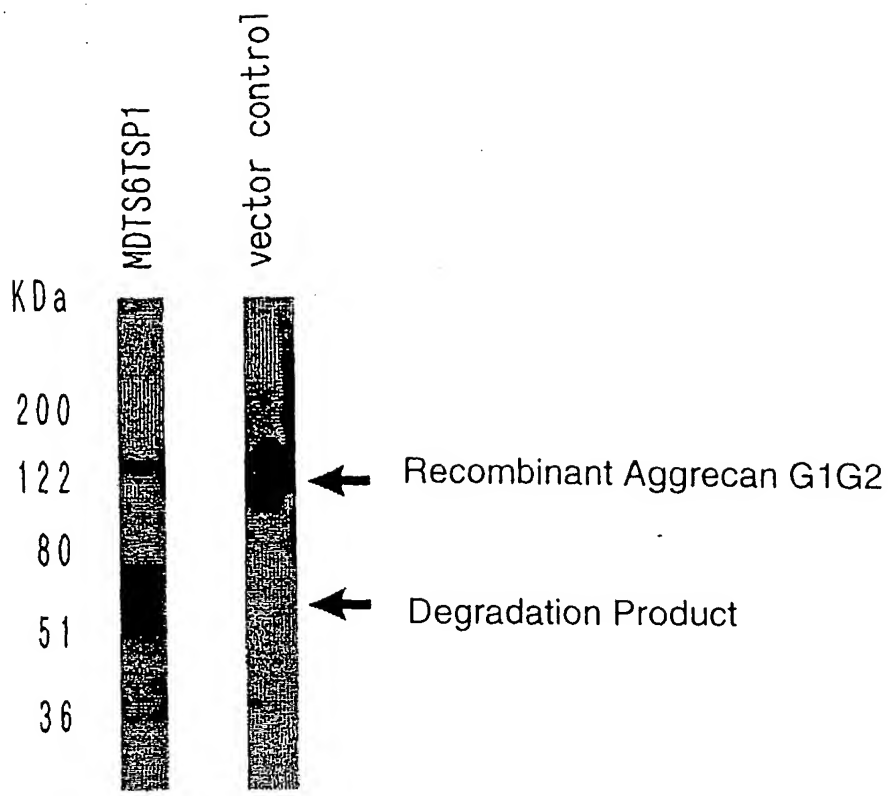
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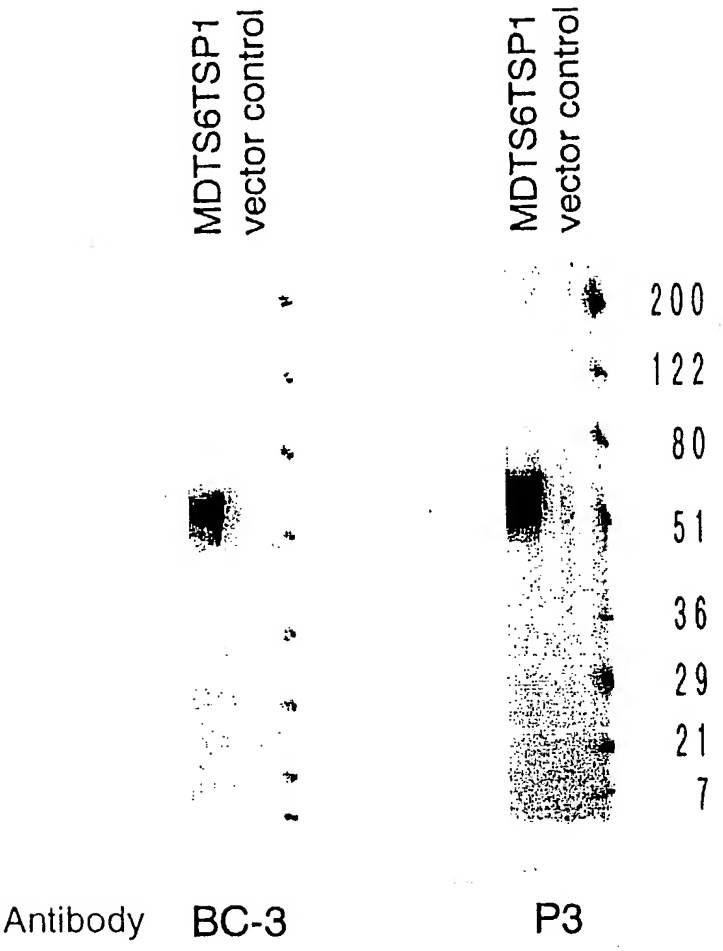
[Fig. 3]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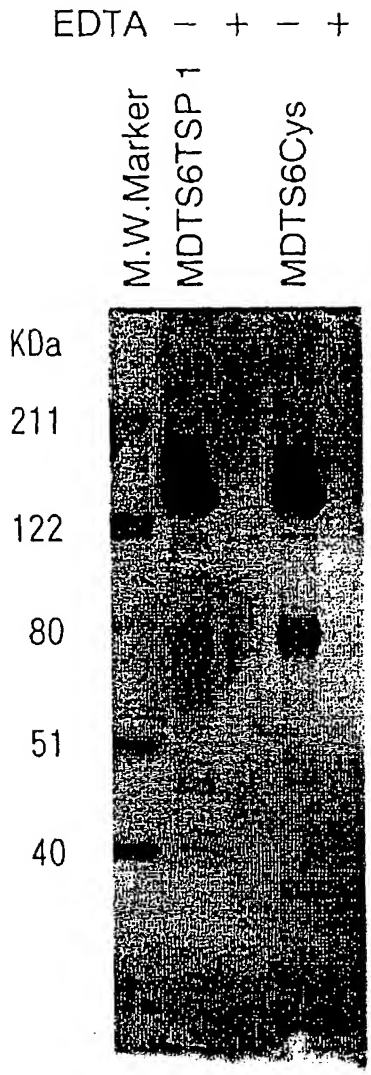
[Fig. 4]

[Fig. 5]



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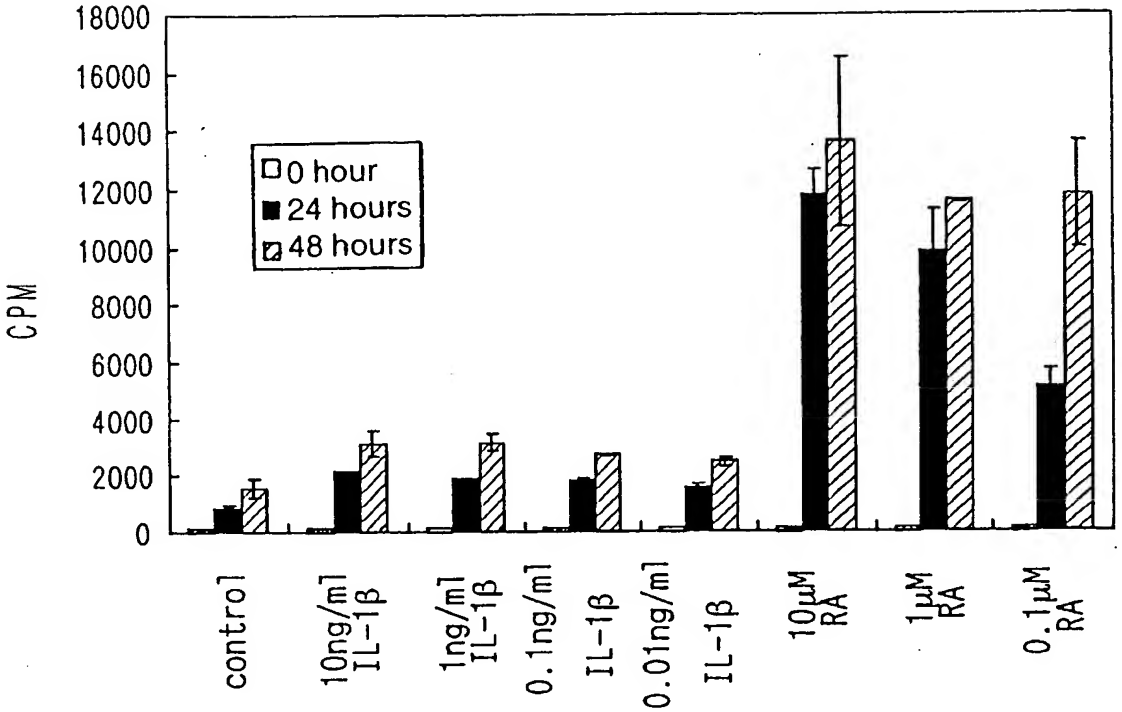

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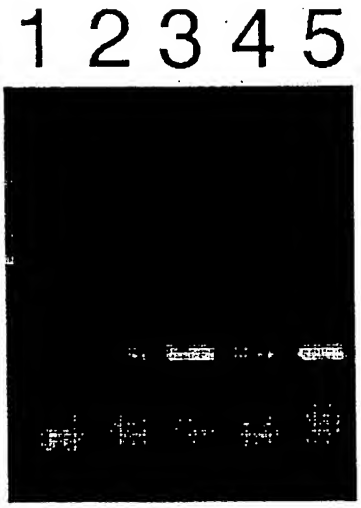

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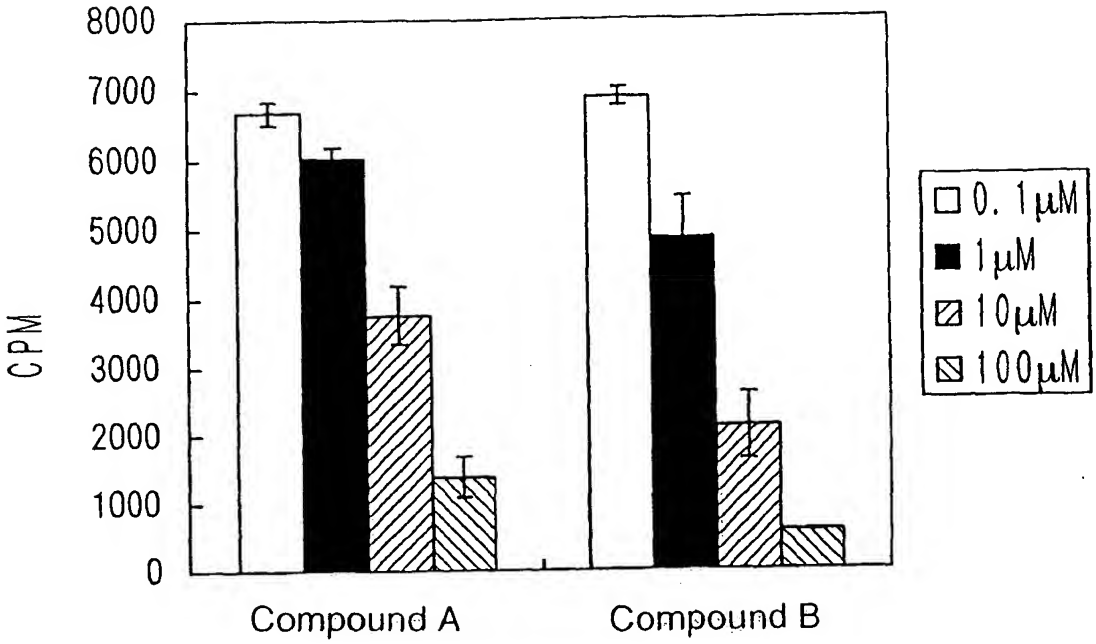
4: IL-1β 6 hours Treatment
5: R.A. 6 hours Treatment

[Fig. 8]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CPM
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1μM
10μM
100μM

Annex

(1) page 10, lines 13-18 of English translation of Japanese Patent Application No. 2000-144020

"... any of a metalloprotease which contains an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and has a protein degradation ability, or a metalloprotease which is an equivalent of the metalloprotease."

(2) page 11, lines 3-9 of English translation of Japanese Patent Application No. 2000-144020

"... an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and has a protein degradation ability, or a metalloprotease which is an equivalent of this metalloprotease."

(3) from page 11, line 10 to page 12, line 15 of English translation of Japanese Patent Application No. 2000-144020

" Regarding the "equivalent of the metalloprotease", (1) in the case of an equivalent of the metalloprotease containing an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 213th position to the 583rd position, and which has the protein degradation ability, (2) in the case of an equivalent of the metalloprotease containing an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 1st position to the 583rd position, and which has the protein degradation ability, or (3) in the case of an equivalent of the metalloprotease containing the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd

position of the amino acid s quence represented by SEQ ID NO:1 and having a protein degradation ability, it is a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in respective sequences, and which has the protein degradation ability. "

(4) page 13, line 8 to page 15, line 21 of English translation of Japanese Patent Application No. 2000-144020

" [0009]

The gene coding for the novel metalloprotease of the invention is any gene which contains a nucleotide sequence coding for the metalloprotease, namely a metalloprotease gene which contains a nucleotide sequence coding for an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 or an equivalent of the metalloprotease and which has a protein degradation ability.

Also, the gene coding for the novel metalloprotein of the invention may be any metalloprotease gene which contains a nucleotide sequence coding for a metalloprotease represented by an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1 or for an equivalent of the

metalloprotease and which has a protein degradation ability.

In addition, it may be any metalloprotease gene which has a nucleotide sequence coding for a metalloprotease represented by the amino acid sequence shown in SEQ ID NO:1, or by an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence shown in SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence shown in SEQ ID NO:1, or for an equivalent of the metalloprotease, and which has a protein degradation ability.

Regarding the "nucleotide sequence coding for an equivalent of the metalloprotease", (1) in the case of a nucleotide sequence coding for an equivalent of the metalloprotease containing an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 213th position to the 583rd position, and which has the protein degradation ability, (2) in the case of a

nucleotide sequence coding for an equivalent of the metalloprotease containing an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in the amino acid sequence of from the 1st position to the 583rd position, and which has the protein degradation ability, or (3) in the case of a nucleotide sequence coding for an equivalent of the metalloprotease containing the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1 and having a protein degradation ability, it is a nucleotide sequence coding for a metalloprotease in which one to several amino acid residues may be substituted, deleted and/or inserted at one to several positions in respective sequences, and which has the protein degradation ability."

(5) page 25, line 20 to page 27, line 20 of English translation of Japanese Patent Application No. 2000-144020

" [0017]

2) Methods for the production of the vector of the invention, the host cell of the invention and the recombinant protein of the invention

The thus isolated fragment containing the gene coding for the novel protein of the invention can be transformed into eucaryotic or procaryotic host cells by again integrating it into an appropriate vector DNA. In addition, it is possible to express the gene in respective host cells by introducing an appropriate promoter and a sequence concerned in the gene expression into these vectors. ..."